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RESEARCH STUDENT	MR. KAUDEWAR DATTAHARI RAJARAM
MAIL ID.	drkaudewar@gmail.com
MOBILE NO.	9975827241 / 9284788131
CATEGORY	ST
SUBJECT	PHARMACY
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RESEARCH GUIDE	DR. NAGOBA S. N.
MAIL ID.	nagobashivraj@gmail.com
MOBILE NO.	9637908901
DESIGNATION	PROFESSOR AND HEAD, DEPARTMENT OF PHARMACEUTICS, CHANNABASWESHWAR PHARMACY COLLEGE (DEGREE), LATUR
RESEARCH CENTRE	CHANNABASWESHWAR PHARMACY COLLEGE (DEGREE), LATUR
UNIVERSITY	SWAMI RAMANAND TEERTH MARATHWADA UNIVERSITY, NANDED-431606, (MAHARASHTRA STATE), INDIA.
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"FORMULATION AND EVALUATION OF NANOCOCHLEATE DRUG DELIVERY SYSTEM"

A SYNOPSIS SUBMITTED FOR THE REGISTRATION OF

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By

MR. KAUDEWAR DATTAHARI RAJARAM
M. Pharm.

Under the Guidance of

DR. NAGOBA S. N.
M. Pharm., Ph.D.

Professor and Head,

Department of Pharmaceutics,

Channabasweshwar Pharmacy College (Degree), Latur.

स्वामी रामानंद तीर्थ
मराठवाडा विद्यापीठ,नांदेड.

Swami Ramanand Teerth Marathwada University,

Nanded-431606, (Maharashtra State), India.

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1. Abstract of proposed work plan/ problem:

Cancer is one of the leading cause of mortality in India and worldwide. As per GLOBOCON 2020 report, around 99,58,133 deaths are recorded only from cancer annually and 1,92,92,789 new cases are seen in the year 2020. Most of the antineoplastic agents which are used for treatment are chemotherapy medicines are administered by parenteral route. These antineoplastic medicines are harmful not only to tumor cells but to the body's regular cells including intestinal epithelial and bone marrow cells which result into severe adverse drug reactions and failure of treatment regimen. Hence, the enhancement of therapeutic index by increasing therapeutic efficacy towards the tumor cells and reducing toxicity to normal tissues is a globally needed strategy to improve safety and efficacy of cancer therapy. Nanotechnology has flashed as a strategy of research interest in cancer treatment due to its capability to resolve a numerous problems associated with drug such as poor solubility in water, absence of aiming capability, systemic toxicity, lack of distribution specificity and less therapeutic index. In last few years, the formulation of rifampicin, amphotericin B, paclitaxel (PTX), fisetin and quercetin nanocochleates was found to be promising and showed a good strategy to improve drugs bioavailability, efficacy and safety in various disease treatments especially in cancer chemotherapy. Based on the review of articles on nanocochleates we found that it's a need of hour to formulate and evaluate anticancer drug as nanocochleates. Anticancer drug-loaded nanocochleates will be prepared from liposomes by trapping or hydrogel method. Such prepared drug loaded nanocochleates will be optimized and evaluated for particle size, zeta potential, entrapment efficacy, PXRD, DSC, TEM, in-vitro drug release, in vivo pharmacokinetic study, in vitro anticancer cell line study and stability studies.

2. Introduction:

A novel lipid based nanoparticulates- A potential drug carriers

In recent years, various nanotechnology platforms in the area of medical biology, including both diagnostics and therapy, have gained remarkable attention. Moreover, research and development of engineered multifunctional nanoparticles as pharmaceutical drug carriers have spurred exponential growth in applications to medicine in the last decade. Number of nanoparticles approach are emerging, including nano-emulsions, dendrimers, nano-gold, liposomes, drug-carrier conjugates, antibody-drug complexes, and magnetic nanoparticles, are primarily based on unique assemblies of synthetic, natural, or biological components, including but not limited to synthetic polymers, metal ions, oils, and lipids as their building blocks. However, the potential success of these particles in the clinic relies on consideration of important parameters such as nanoparticle fabrication strategies, their physical properties, drug loading efficiencies, drug release potential, and, most importantly, minimum toxicity of the carrier itself. Among these, lipid-based nanoparticles bear the advantage of being the least toxic for in vivo applications, and significant progress has been made in the area of DNA/RNA and drug delivery using lipid-based nanoassemblies.

A growing number of pharmaceutical companies' drug pipelines are being filled with water-insoluble (BCS II/IV) or low permeable (BCS III/IV) therapeutic entities. Lipid-based drug delivery systems have become an important tool for the formulation development of those compounds.

Lipid-based drug delivery systems have become a technology of choice because they can enhance the oral bioavailability of drugs by increasing dissolution and solubility by pre-dissolving drugs in lipid carriers improving drug permeability in the gastrointestinal tract (GI) by inhibition of P-gp and other efflux transporters, bypassing the first-pass metabolism of the drug through the lymphatic absorption processes. There are other benefits to lipid-based drug delivery systems. For one, they have the potential to decrease the food-effect and increase reproducibility of the pharmacokinetic (PK) profile of orally administered drugs by reducing erratic absorption.

Nanocochleates- A novel lipid based nanoparticulate drug delivery

Nanocochleate a liposome derived drug delivery system represent a unique approach consisting of entrapment of desired drug molecule into multilayered assemblies of negatively charged phospholipids sheet rolled up in spiral shape and a divalent cation with no internal aqueous phase. Figure 1 shows the schematic representation of cochleate formulation. Cochleates differ from liposomes in having water-free interior, rodshape & rigid stable structure. These unique properties of cochletes make it suitable for delivery of mainly hydrophobic drugs having poor bioavailability like doxorubicin, amphotericin B and clofazimine. Thus it provides a potential delivery system for the wide class of drugs as proteins and peptides, anticancer agent, antiviral agent, anaesthetic, steroidal anti-inflammatory agent, immunosuppressant and tranquilizer etc.

Dr. Dimitrious Papahadjoupoulos and his co-workers in 1975 first discovered Cochleates as precipitates formed by the interaction of negatively charged phosphatidylserine and calcium. This was cylindrical in shape and he named these cylindrical structures as "cochleate" means rolled lipid shell. Cochleates are made up of a series of solid layers and devoid of water so its exposure to harsh environmental conditions or enzymes doesn't affect encapsulated drug molecules and kept it intact within the interior.

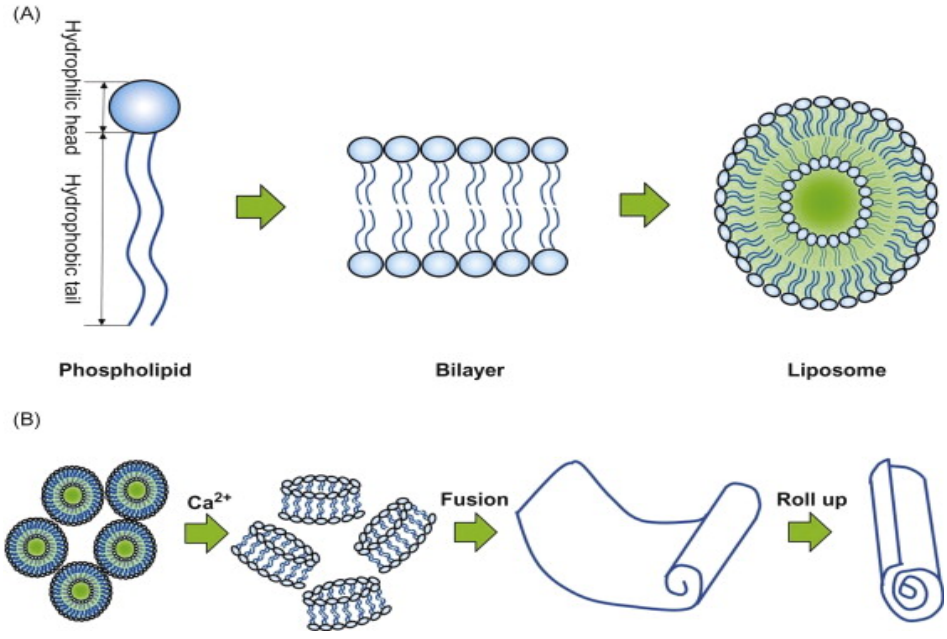

Figure 1: Schematic representation of Cochleate formulation

Basics of Cochleate and Nanocochleates

Cochleate and nanocochleates are novel lipoidal drug delivery system which represent a new approach suitable for the administration of a wide range of therapeutics including drugs, genes, and vaccine antigens. Cochleates have a peculiar multilamellar structure which imparts them extraordinary stability. Interaction of negatively charged bilayers with cations result in substantial structural rearrangement that causes aggregation and fusion of bilayers. This interaction triggers conversion of aliphatic acyl chains in lipid bilayer into gel state. The resulting planar bilayer sheets consist of lipid and binding agent. These crystallized planar sheets further tightly fold upon to form cylindrical carpet roll like structure. Hence a typical cochleate is made up of sheets consisting alternating layers of binding agent and lipid bilayer. Nanocochleates are cylindrical (cigar-like) microstructures that consist of series of lipid bilayers. Nanocochleate delivery vehicle are stable phospholipid-cation precipitates composed of simple naturally occurring material like polyphosphotidylserine and calcium. They posses little or no aqueous internal space between them.

Both cochleate and nanocochleate structure is a series of solid layers so that, even if the outer layers of cochleate and nanocochleate are exposed to harsh GI conditions or enzymes, the encapsulated drug molecules will remain intact within the interior. Nanocochleates contains both hydrophilic and hydrophobic surface which makes it more suitable for encapsulation of both hydrophobic drugs like amphotericin B and clofazimine and amphipathic drugs like doxorubicin. Nowadays, oral route remains the alternative way for administrating therapeutic agents. In particular, lipid-based nanocochleate delivery system appears to provide answers to oral delivery challenges by formulating different kinds of biological molecules including genes, proteins and peptides and vaccines, antigens and drugs, especially hydrophobic once that were not having oral bioavailability. Nanocochleates are stable lipid-based delivery formulations whose structures and properties are very different from liposomes. Nanocochleates are unique platform technology for the delivery of with ranges of drugs and molecules such as proteins and peptides, polynucleotides. Thus, it is a potential drug delivery system for the wide class of drugs.

Components of Nano-cochleate Drug Delivery System

The three major components used in the preparation of nanocochleates are active pharmaceutical ingredient (API), lipids, and cations.

1. API used may be hydrophilic or hydrophobic and or can be used both.
2. Lipids used will be as follow: Phosphatidyl serine (PS), phosphatidic acid (PA), di-oleoyl PS, phosphatidylinositol (PI), phosphatidyl glycerol (PG), phosphatidyl choline (PC), dimyristoyl PS, phosphatidyl ethanolamine (PE), di-phosphatidyl glycerol (DPG), dioleoyl phosphatidic acid, di-stearoyl phosphatidyl serine, di-palmitoyl PG, etc.
3. Cations: Zn^{+2} or Ca^{+2} or Mg^{+2} or Ba^{+2}, etc.

Advantages of Nanocochleates

1. Nanocochleate are more stable than liposomes because of less oxidation of lipids and water free inner core.
2. They exhibit the efficient incorporation of hydrophobic drugs into lipid bilayers of nanocochleate structure. They can also exhibit efficient incorporation of antigens with hydrophobic moieties into lipid bilayers of nanocochleate structure.
3. With nanocochleate, intravenous drugs can be administered orally. For example, amphotericin B, a potent antifungal.
4. Nanocochleated drug delivery reduces the stomach irritation and other side effects seen in conventional drug delivery. They protect the encapsulated drug from degradation avoiding exposure to sunlight, oxygen, moisture and temperature.
5. The components of lipid bilayer composed of simple lipids, that are naturally occurring and makes nanocochleates a safe and biocompatible delivery vehicles.
6. Nanocochleated drug delivery improve the oral bioavailability of a broad spectrum of compounds, such as those with poor water solubility and proteins and peptides, biopharmaceuticals, which have been difficult to administer. For example, Ibuprofen for arthritis.
7. They are produced easily and safely.

3. Review of Literature and Development in the subject (Previous work done in the relevant area):

1. **Min Liu. et al. (2019)** Nanocochleates without adding ions were produced. Chitosan played the role in inducing coiling of negatively charged lipids to form nanocochleates with an mean size of 114.2 ± 0.8 nm, without the use of calcium ions. These functional chitosan-induced nanocochleates augmented gastrointestinal absorption of cyclosporine A and manifested up to a 3-fold increase in oral bioavailability. Fluorescence-labelling study demonstrated, absorption mainly occurred in the duodenum and jejunum. Transport studies revealed that uptake of chitosan-induced nanocochleates by Caco-2 cells occurred through endocytosis mediated by clathrin- and caveolae. Furthermore, three cellular tight junction proteins, ZO-1, F-actin and claudin-4, were significantly down-regulated. This implies chitobiose-induced nanocochleates are able to reconstruct and open tight junctions in intestinal epithelial cells to enhance drug absorption.
2. **Ishwor Poudel et al (2018)** developed biotinylated chitosan (BI-CHI) decorated docetaxel (DTX) loaded nanocochleates (BI-CHI-DTX-NC) to achieve controlled drug release, improve bioavailability, targeted delivery and enhanced anticancer potency with the reduced systemic toxicity of DTX. The development involved the loading of DTX to nanocochleates (DTX-NC) through conversion of dimyristoylphosphati-dylglycerol-sodium (DMPG-Na) and cholesterol bearing liposome on addition of calcium ions, followed by encapsulated DTX-NC with BI-CHI (BI-CHI-DTX-NC) and compared with DTX and DTX-NC. The release of DTX indicated strong pH dependence and implies strong hydrogen-bonding between nanocochleates and DTX. Formulated BI-CHI-DTX-NC demonstrated higher in-vitro anticancer activity in biotin over expressed human breast cancer MCF-7 cells. The targeting effect for the BI-CHI-DTX-NC was also demonstrated. The concentration of the drug needed for growth inhibition of 50% of cells in a designed time period (GI50) was 1.8 lg/ml for free DTX while it was decreased by 33.34% for the DTX-NC (1.2 lg/ml). Furthermore, the GI50 value of BI-CHI-DTX-NC was 0.2 lg/ml, i.e. an 88.89% decrease was observed as compared to DTX solution. Moreover, bioavailability of DTX from BI-CHI-DTX-NC was increased by 10-folds with longer

circulation time and slower plasma elimination with low tissue distribution as compared to DTX solution. The results indicate that the BI-CHI-DTX- NC has the potential to be applied for targeting anticancer drug delivery.

3. **S A Sonawane et al (2017)** prepared quercetin loaded nanocochleates by a trapping method and were evaluated for size, entrapment efficiency, in vitro quercetin release, cytotoxicity study. Stable rolled-up layers as well as a tubular structure of nanocochleates possessing particle size and encapsulation efficiency of 180 ± 3 nm and 76.69 ± 3.21%, respectively were obtained. Nanocochleates demonstrated sustained release of quercetin at physiological pH. A significant improvement in vitro anticancer efficacy towards human breast cancer MCF-7 cells was observed.
4. **Beatriz et al (2017)** formulated nanocochleates of essential oil from Artemisia absinthium L. (EO-Aa). Asteraceae in nanocochleates (EO-Aa-NC) was evaluated in vitro against intracellular amastigotes of L. amazonensis and non-infected macrophages from BALB/c mice. In addition, the EO-Aa-NC was also evaluated in vivo against on experimental cutaneous leishmaniasis, which body weight, lesion progression, and parasite load were determined. EO-Aa-NC displayed IC 50values of 21.5±2.5µg/mL and 27.7±5.6µg/mL against intracellular amastigotes of L. amazonensis and non-infected peritoneal macrophage, respectively. In the animal model, the EO-Aa-NC (30 mg/kg/intralesional route/every 4 days 4 times) showed no deaths or weight loss greater than 10%. In parallel, the EO-Aa-NC suppressed the infection in the murine model by approximately 50%, which was statistically superior ($p < 0.05$) than controls and mice treated with EO-Aa. In comparison with Glucantime®, EO-Aa-NC inhibited the progression of infection as efficiently ($p > 0.05$) as administration of the reference drug.
5. **Akash Yadav et al (2016)** developed the formulation of nanocochleates of rifampicin for improving its absorption through instestinal mucosa. Permeability studies of the cochleate formulation of rifampicin shows more than two fold increase in the apparent permeability over normal rifampicin. The apparent permeability of rifampicin was found to be 3.56 × 10-6 cm/s. Formulation of drug as nanocochleates increased its absorption through the instestinal mucosa. This resulted in a significant increase in the apparent permeability to 7.78 ×10-6 cm/s. Hence the presented work proves a marked

increase in permeability of rifampicin through small instestine by its formulation as nanocochleates, which is a good strategy to overcome the problem of malabsorption of rifampicin in presence of other anti-TB drugs as well as food and antacid, thus lowering the dose and side effects of the drug and requires further clinical studies to confirmation. It was observed that the cochleate formulation of rifampicin shows significant increase in apparent permeability.

6. **A P Pawar et al. (2014)** developed PTX-loaded nanocochleates (PTXNC) by addition of calcium ion into preformed nanoliposomes (PTXNL) comprising PTX, phosphatidylcholine and cholesterol and evaluated by in vitro and in vivo methods in comparison with PTXNL and pure PTX. Based on the clinical fact that paclitaxel (PTX) intravenous injection frequently causes hypersensitive reaction, its oral administration is vital for its clinical development. However, the development oral PTX formulation is difficult due to its low oral bioavailability caused by its low permeability, low solubility, efflux and affinity for intestinal and liver metabolic enzymes. In an effort to develop an alternative formulation of PTX suitable for oral administration, PTX-loaded nanocochleates (PTXNC) were developed by addition of calcium ion into preformed nanoliposomes (PTXNL) comprising PTX, phosphatidylcholine and cholesterol and evaluated by in vitro and in vivo methods in comparison with PTXNL and pure PTX. Stable tubular rod structure of PTXNC possessing particle size, encapsulation efficiency and zeta potential of 269 nm, 71.12 % and -3.9 mV, respectively were obtained from homogenous unilamellar, discrete and spherical structured nanoliposomes with diameter and zeta potential of 247 nm and -12.3 mV, respectively. Powder X-ray diffraction and thermal study revealed that PTX was partial crystalline and amorphous state in PTXNL and PTXNC, respectively. PTXNC demonstrated controlled release of PTX. PTXNC showed many fold improvement in vitro anticancer activity towards A-549 (human lung adenocarcinoma cells), ovarian cancer OVCAR-3 and breast cancer MCF-7 cells with a 14- and 19-fold increase in oral bioavailability in rats as compared to PTXNL and pure PTX, respectively. Moreover, PTXNC showed low tissue distribution. These results collectively suggest that nanocochleates could therefore advantageously be employed

to improve anticancer activity of PTX and an alternative to the present intravenous administration.

7. **Bothiraja et al (2013)** developed and evaluated fisetin-loaded nanocochleates to improve its therapeutic efficacy. Using the trapping method, fisetin-loaded dimyristoylphosphatidylcholine liposomal vesicles were converted into nanocochleates by the action of Ca2+ ions. These nanocochleates were further evaluated for physicochemical, in vitro anticancer and haemolysis, pharmacokinetics and tissue distribution study in mice. Stable rolled-up layers as well as elongated structure of nanocochleates possessing particle size and encapsulation efficiency (EE) of 275 ± 4 nm and 84.31 ± 2.52%, respectively were obtained. Nanocochleates demonstrated safety and a sustained release of fisetin at physiological pH. A 1.3-fold improvement in vitro anticancer towards human breast cancer MCF-7 cells was observed. Pharmacokinetics studies in mice revealed that nanocochleates injected intraperitonially showed a 141-fold higher relative bioavailability. Moreover, a low tissue distribution was observed.
8. **Pawar et al (2013)** studied phosphatidylserine based cochleate formulation of ketoconazole. 3 by 2 factorial design used to optimize cochleate formulation and to study effect of phosphatidylserine and drug on the properties and performance of cochleates. Cochleates were also characterized by FTIR and DSC. The antifungal activity and stability of KCZ cochleates too was investigated. Cochleates of size 0.282 μm ± 0.05 to 72.52 μm ± 2.2 and entrapment efficiency of 57.86% ± 4.55% to 97.27% ± 2.77 were obtained. The variables of the 32 factorial design significantly affected the cochleate size. Cochleates demonstrated promising role in topical delivery of drugs as the small sized cochleates caused significant release across the skin while the larger ones were retained in the skin leading to drug accumulation therein. Antifungal activity testing confirmed the preservation of antifungal activity by the encochleated drug. Nevertheless, the prepared cochleate formulations possessed stability profile similar to liposomal counterpart.
9. **Santangelo et al (2000)** prepared cochleates containing Amphotericin B (CAMB) inhibit the growth of Candida albicans, and the in vivo therapeutic efficacy of CAMB administered orally was evaluated in a mouse model of systemic candidiasis. The

results indicate that 100% of the mice treated at all CAMB doses, including a low dosage of 0.5 mg/kg of body weight/day, survived the experimental period (16 days). In contrast, 100% mortality was observed with untreated mice by day 12. The fungal tissue burden in kidneys and lungs was assessed in parallel, and a dose-dependent reduction in C. albicans from the kidneys was observed, with a maximum 3.5-log reduction in total cell counts at 2.5 mg/kg/day. However, complete clearance of the organism from the lungs, resulting in more than a 4-log reduction, was observed at the same dose. These results were comparable to a deoxycholate AMB formulation administered intraperitoneally at 2 mg/kg/day ($P < 0.05$). Overall, these data demonstrate that cochleates are an effective oral delivery system for AMB in a model of systemic candidiasis.

4. Objectives of Research/ Proposed Hypothesis:

Aim: Formulation and Evaluation of Nanocochleate Drug Delivery System.

Objectives of the Research Work:

- To formulate, optimize and characterize anticancer drug-loaded nanoliposomes and nanocochleates.
- To improve the efficacy, drug loading capacity and biocompatibility of anticancer drug by formulating in nanocochleates.
- To minimize the toxicity and increase the stability of anticancer drug by encapsulating in nanocochleates system.
- To evaluate anticancer potential of formulation by in-vitro cell culture study.
- To study In-vivo anticancer activity of end product.
- To evaluate in-vivo bioavailability of drug from formulation in comparison with pure drug in experimental animal.
- To study the tissue distribution of drug from formulation in comparison with pure drug in experimental animal.

5. Materials and Methodology to be adopted:

1. **Literature review**

2. **Selection and Collection of anticancer drug & excipients**

   Selection of anticancer drug: e.g. Capacitabine or Gemcitabine etc.

   Selection of a lipid: To formulate the nanocochleates, one of the following lipid will be selected e.g. Phosphatidylserine or Phosphotidylethanolamine or Phosphotidylcholine or Phosphatidylinosotol etc.

3. **Experimental work**

   A. **Preformulation studies**
      1. **Spectroscopic study**
      2. **Solubility study**
      3. **Crystallinity study**

   B. **Compatibility study**

4. **Formulation development**

   Step I: Preparation of drug loaded nanoliposomes

   Liposomes will be prepared by ethanol injection method or rotary evaporator method or any other suitable method.

   Step II: Preparation of drug loaded nanocochleates

   Nanocochleates of above prepared nanoliposomes will be prepared by trapping or hydrogel method or any other suitable method.

5. **Characterization of drug-loaded Nanoliposomes and Nanocochleates**
    1. Yield and drug content
    2. Particle size and zeta potential
    3. Encapsulation efficiency
    4. Surface morphology by Transmission electron microscopy (TEM)
    5. Fourier transform infrared spectroscopy
    6. XRD study
    7. Differential scanning colorimetry
    8. In vitro drug release

6. **Stability studies**
7. **In vitro anticancer cell line study**
8. **In vivo pharmacokinetic study**
9. **In vivo anticancer study**

6. Importance of study/ Society application:

- **International status:**
- Nanomedicines have become well appreciated in recent times due to the fact that nanostructures could be utilized as delivery agents by encapsulating drugs or attaching therapeutic drugs and deliver them to target tissues more precisely with a controlled release. Nanomedicine, is an emerging field implementing the use of knowledge and techniques of nanoscience in medical biology and disease prevention and remediation. It implicates the utilization of nanodimensional materials including nanorobots, nanosensors for diagnosis, delivery, and sensory purposes, and actuate materials in live cells. This is very true in diseases like cancer where there are many challenges. Worldwide there is increasing number of cancer patients, consequently anticancer drug use also increased. Most of the studies in last few years with nanoparticulates showed a promising result and accordingly there is increasing number of patents on nano drug delivery in chemotherapy of cancer.
- **United States patent US6153217A (28/11/2000):** Nanocochleate formulations, process of preparation and method of delivery of pharmaceutical agents.

- **National status:** India being a second largest populated country in the world and is facing a many more challenges in cancer therapy. Increased number of cancer patients in India due to smoking, sedentary life style, poor living standard, pollution, lack of awareness on cancers early signs and symptoms etc. A big issue in India is lack of availability of most effective, safe and economic drug delivery for cancer chemotherapy. Nanoparticulates, especially nanocochleates have the potential to overcome the challenges related to cancer chemotherapy. There is huge scope in India to develop a drug delivery with improved efficacy, biocompatibility, biodistribution and minimized toxicity in cancer chemotherapy.
- **Indian patent application no. 2190/MUM/2011 (18/11/2020):** A nanocochleate-nanosphere complex.

►Significance of the study:

One of the drug delivery system is the lipid based nanoparticulate system which bears potential for the development of new pharmaceuticals due to its many advantages such as biocompatibility, increased drug loading ability, ease and safety of production, reduced side effects and improved efficacy. The use of lipid based nanoparticulate system has shown improved bioavailability with reduced toxicity in various synthetic drugs like doxorubicin and various phytoconstituents like fisetin, taxanes. Lipid based nanoparticulate system i.e. nanocochleates are resistant to external oxidation and protect the active moiety in biological fluids due to the series of solid layers.

The scope of lipid based nanoparticulate system is huge in research with number of poorly soluble as well as poorly permeable drugs belonging category BCS-class II and or BCS-class IV having many formulation challenges. The challenges arise due to poor bioavailability which is due to solubility and absorption limitations. One of key to BCS class II and IV drug's improved physiochemical absorptions lies in lipid based formulations viz; liposomes, nanocochleates etc. In recent studies nanocochleates loaded with anticancer pharmaceuticals showed improved bioavailability, biocompatibility, efficacy, drug loading capacity and decreased toxicity. Hence it is an need of developing countries where cancer occurrence is more by virtue of poor state of life (diet, hygiene); to develop a formulation of the anticancer drugs in nanocochleate system.

7. Proposed work Plan/ Formulation and Structure of Study:

Year-wise Plan of work and targets to be achieved:

Sr. No.	Expected target to be achieved	1-6 months	7-10 months	11-22 months	22-27 months	28-33 months	34-36 months
01	Literature survey	Literature survey					
02	Selection and collection of drug and excipients	Selection and collection of drug and excipients					
03	Formulation, development and optimization	Formulation, development and optimization					
04	Evaluation of nanocochleates	Evaluation of nanocochleates					
05	In vitro and in vivo study	In vitro and in vivo study					
06	Statistical analysis, data interpretation and conclusions	Statistical analysis, data interpretation and conclusions					
07	Publishing research and submitting data	Publishing research and submitting data					

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Name of Research Candidate: Mr. Kaudewar Dattahari Rajaram	**Signature:**
Remarks of The Guide	Satisfactory
Name and Designation of Research Supervisor: Dr. S. N. Nagoba Professor and Head, Department of Pharmaceutics, Channabasweshwar Pharmacy College (Degree), Latur.	Dr. Nagoba S. N. Professor & Head, Department of Pharmaceutics, Channabasweshwar Pharmacy College (Degree), Latur